TRANSLATION OF TYMV RNA INTO HIGH MOLECULAR WEIGHT PROTEINS

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1. Introduction

The wheat germ cell-free system leads to the translation of TYMV RNA into mature coat protein and several other polypeptides [1], although not to a polypeptide whose size corresponds to the full translation of the viral genome.

TYMV contains not only complete viral genomic RNA (2×10^6 daltons), but also several other classes of RNA molecules ranging from $2–0.25 \times 10^6$ daltons [2,3]. The complete viral genome by itself is infectious [2], and therefore contains all the information necessary to generate new virus particles; in a wheat germ cell-free system, however, it directs the synthesis of only small amounts of coat protein molecules, if at all, thereby suggesting that the coat protein gene is masked in such a system.

In order to determine whether TYMV RNA can direct the synthesis of high molecular weights proteins (>100 000 daltons), we turned to the use of the mRNA-dependent reticulocyte lysate which has been shown by Pelham and Jackson [4] to translate TMV RNA into one protein of 130 000 daltons.

Our results show that in the reticulocyte system total TYMV RNA is efficiently translated into two high molecular weight proteins, one of 195 000 daltons, the other of 150 000 daltons, as well as into coat protein (20 000 daltons). The largest protein is equivalent to the size of the genomic RNA, minus the coat protein gene and the 'tRNA-like' structure adjacent to this gene [5,6]. The analysis of the proteolytic digests of both high molecular proteins indicates that the protein of 150 000 daltons corresponds to the translation in phase of most of the RNA coding for the 195 000 dalton protein.

Abbreviations: TYMV, turnip yellow mosaic virus; TMV, tobacco mosaic virus; EGTA, ethylene-glycol-bis (2-amino-ethyleter)*N,N'*-tetraacetic acid; PPO, 2,5-diphenyloxazole

This paper is gratefully and respectfully dedicated to Professor Edgar Lederer on the occasion of his 70th anniversary

2. Materials and methods

TYMV was extracted from infected Chinese Cabbage leaves (the kind gift of S. Astier, INRA, Versailles) as in [1]. L-[^{35}S]Methionine (700 Ci/mmol) was from Amersham, *N*-acetylphenylhydrazine and hemin from Sigma, micrococcal nuclease from Boehringer, *S. aureus* (V_8 protease from Miles Laboratories and rat liver tRNA from General Biochemicals. RP Royal X-O-mat Kodak films were used for autoradiography.

2.1. *TYMV RNA preparation*

TYMV RNA was extracted from the virion using the phenol, chloroform, SDS method, essentially as in [7]. TYMV at 5 mg/ml was mixed with equal vol. 100 mM Tris–HCl, pH 7.5-saturated phenol–chloroform (1:1) containing 2% SDS, and stirred at room temperature for 20 min. After centrifugation the aqueous phase was collected, mixed with equal vol. chloroform, stirred again and re-extracted. The RNA solution was then made 0.2 M with Na acetate, pH 5, and precipitated with 2 vol. ethanol. After centrifugation the RNA pellet was dried, dissolved in 10 mM Hepes–KOH, pH 7.5, at the desired concentration and stored at −80°C.

2.2. *Preparation of an mRNA-dependent reticulocyte lysate*

Rabbits were made anaemic using *N*-acetylphenylhydrazine, and the reticulocyte lysate prepared as in [8]. The lysate was then treated by micrococcal nuclease according to the procedure in [4] with a few minor changes. To 15 ml reticulocyte lysate were added: 1 ml 1.25 mM hemin adjusted to pH 7.5; 7 ml solution containing 70 mM Hepes–KOH, pH 7.5; 35 mM DTT; 3.5 mM calcium chloride; 70 mM creatine phosphate; 350 μg/ml creatine phosphokinase; and 70 μg/ml staphylococcal nuclease. The mixture was incubated 15 min at 20°C and the reaction stopped by adding, at 0°C, 1 ml 0.1 M EGTA adjusted to pH 7.5. The lysate which contained no residual endogenous translational activity was fractionated and stored in liquid nitrogen.

2.3 *In vitro translation of TYMV RNA*

Total protein synthesis was optimized for RNA, magnesium and potassium ion concentrations. Incubations of 50 μl contained: 25 μl mRNA-dependent lysate; 1 mM $MgCl_2$; 100 mM KCl; 25 μM each of the 19 unlabelled amino acids; 0.2 μM L-[^{35}S]methionine; 3 μg rat liver tRNA; and 2.5 μg total TYMV RNA. The incubation was performed at 30°C for 1 h. Total incorporation was checked on a 2 μl aliquot using the hot trichloroacetic acid (TCA) treatment on Whatmann 3 MM disks, and the nature of the translation products was analyzed by 12.5% polyacrylamide–0.1% SDS slab-gel electrophoresis together with protein markers, followed by staining and autoradiography as in [1].

2.4. *Proteolytic digestion of high molecular weight proteins*

To have sufficient labelled material, in vitro protein synthesis was scaled up 4-fold and the products separated on a 15% polyacrylamide–0.1% SDS slab-gel. After electrophoresis the gel was dried without previous staining and autoradiographed. The proteins of 195 000, 150 000 and 20 000 daltons were eluted by excision from the dried gel of the corresponding radioactive bands and incubation in minimal vol. 125 mM Tris–HCl, pH 6.8, 1 mM EDTA and 0.1% SDS for 24 h at room temperature.

Aliquots containing equal amounts of radioactivity were then digested with various concentrations of V_8 protease for 30 min at 30°C as in [9]. The samples were boiled for 2 min, made 5% with respect to glycerol and layered over a 15% polyacrylamide–SDS gel. After electrophoresis, the gel was stained, treated

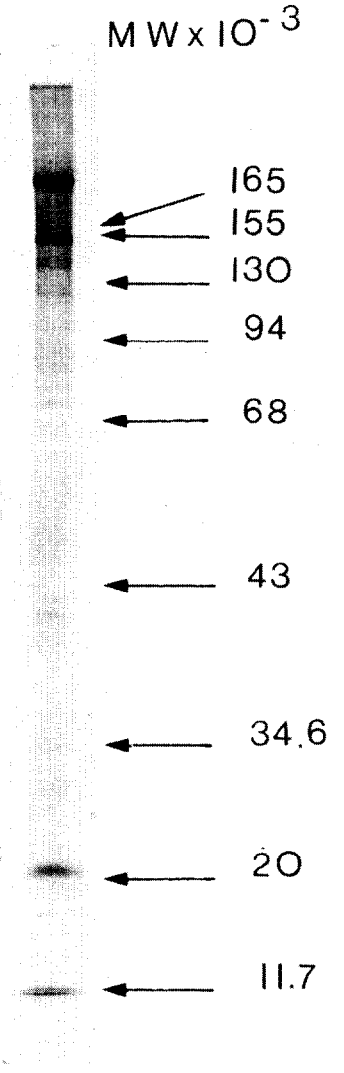

Fig.1. Autoradiogram of the products synthesized in vitro in an mRNA-dependent reticulocyte lysate in response to the addition of TYMV RNA and run on a 12.5% polyacrylamide–0.1% SDS gel. Protein markers run on the same gel are indicated by arrows (β and β′ subunits of *E. coli* RNA polymerase, 165 000 daltons and 155 000 daltons; β galactosidase, 130 000 daltons; phosphorylase a, 94 000 daltons; bovine serum albumin, 68 000 daltons; ovalbumin, 43 000 daltons; carboxypeptidase a, 34 600 daltons; TYMV coat protein, 20 000 daltons; cytochrome *c*, 11 700 daltons.

with dimethylsulfoxide and PPO as in [10], dried and autoradiographed at −70°C using a flash-activated film as in [11].

3. Results

3.1. *Translation of TYMV RNA*

Addition of TYMV RNA to the mRNA-dependent reticulocyte lysate stimulates the incorporation of amino acids into proteins more than 100-fold. Optimal incorporation is obtained with RNA concentrations of 25–50 μg/ml (C. B. and A-L., to be published). After the 1 h incubation, the sample was treated as described in section 2 and layered over a 12.5% polyacrylamide–SDS slab gel and electrophoresed. The result of the autoradiography is presented in fig.1.

TYMV coat protein (20 000 daltons) is synthesized as well as two high molecular weight proteins whose sizes correspond to 195 000 daltons (protein A) and 150 000 daltons (protein B) as judged by the size of the protein markers. In addition diffuse bands between the high molecular weight proteins and the coat protein can be detected which probably correspond to degradation products.

The question raised by these results is the following: are the two HMW proteins different or do they bear common amino acid sequences?

3.2. *Structural similarities between the two high molecular weight proteins*

To answer this question the two radioactive high molecular weight proteins were eluted from the gel, submitted to the action of V_8 protease and the resulting peptides mapped according to the technique introduced in [9] and described in section 2. The results of the autoradiography are shown in fig.2.

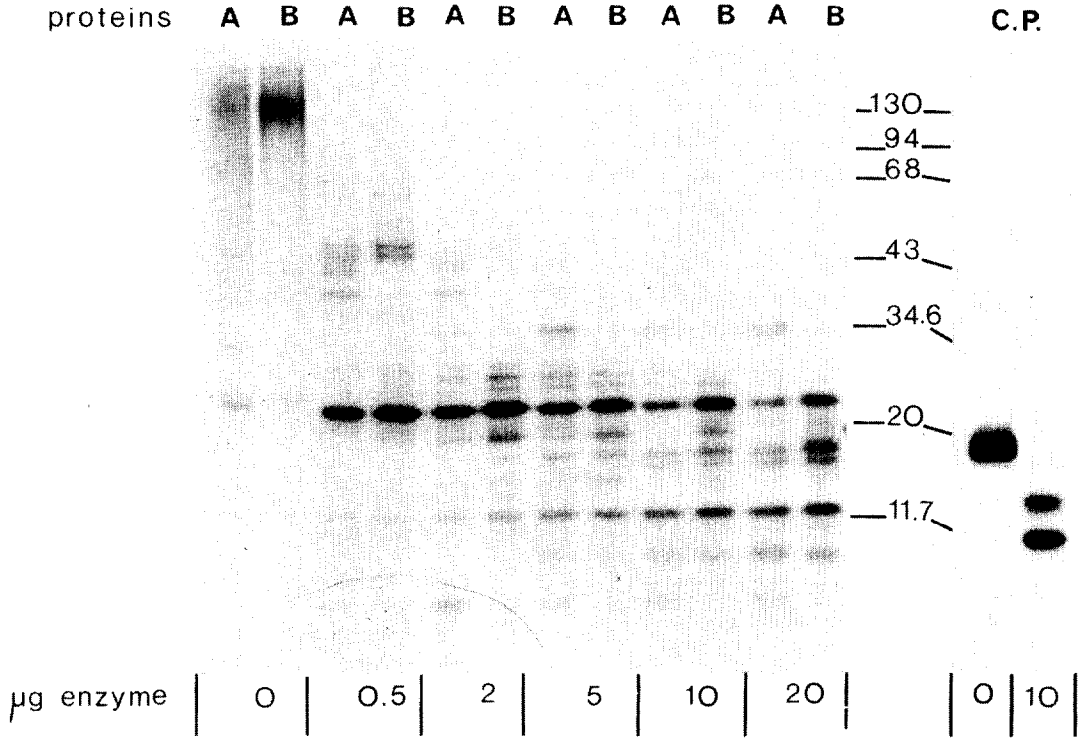

Fig.2. Analysis of the digestion products of proteins A and B. Proteins A and B were eluted from a gel similar to the one presented in fig.1 and aliquots (10 000 cpm) were digested with increasing amounts of V_8 protease (0–20 μg in 50 μl incubation mixtures) and run on a 15% polyacrylamide–0.1% SDS gel. TYMV coat protein (C.P.; 10 000 cpm) eluted in the same conditions was incubated without or with 10 μg V_8 protease. Protein markers are the same as in fig.1.

As the protease concentration is increased, the high molecular weight proteins are hydrolyzed to yield first partial degradation products, and finally, smaller stable peptides. Only a few differences appear between the peptide patterns of proteins A and B thus suggesting that these two proteins possess common amino acid sequences. In the absence of protease, proteins A and B eluted from the preparative gel at 20°C for 24 h are slightly degraded but the resulting peptide profile does not correspond to the profile obtained in the presence of V_8 protease.

Figure 2 also shows a control of TYMV coat protein incubated without or with V_8 protease in the same conditions as those used for proteins A and B. When no protease is added only the protein of 20 000 daltons corresponding to the coat protein appears, but in the presence of V_8 protease, the 20 000 dalton polypeptide disappears completely and gives rise mainly to two smaller products. In no case do these products comigrate with the degradation peptides of proteins A or B. This means that TYMV coat protein is not synthesized as a large precursor (195 000 daltons) which undergoes post-translational cleavage, and that the high molecular weight TYMV RNA possesses two cistrons: the coat protein cistron [2] and another cistron which codes for a protein of 195 000 daltons corresponding to about 90% of the total length of the RNA. The origin of protein B is as yet undefined; experiments are in progress to determine whether it derives from protein A by premature termination or by proteolytic cleavage. We can further not exclude that proteins A and B are initiated in phase at two different sites of the RNA, leading to two large proteins having common sequences.

4. Discussion

The results presented here show for the first time that in the reticulocyte lysate TYMV RNA is translated, in addition to the coat protein, into two high molecular weight products which differ by 45 000 daltons but have common amino acid sequences. The genomic RNA of 2×10^6 daltons (or about 6000 bases) contains at its 3′-end an untranslated region of 108 bases, the 'tRNA-like' region, which is immediately preceeded by the coat protein gene (570 bases) [5,6]. Therefore the remaining coding capacity of the viral genome (5300) is equivalent to a protein of about 190 000 daltons. Protein A corresponds to this coding capacity.

Since we have been unable to isolate a complete genomic RNA which does not direct the synthesis of some coat protein, we do not know if this coat protein is synthesized only from the 0.25×10^6 dalton RNA species as concluded [3] or also from the genomic RNA. Our results might be due to a contamination of the genomic RNA by the coat protein mRNA and a higher affinity of the ribosomes for the 0.25×10^6 dalton RNA initiation site (C. B. and A.-L. H. to be published). Two other possibilities are:

1. During incubation, the genomic RNA is cleaved giving rise to a molecule with an open initiation site for coat protein synthesis.

2. After termination of the synthesis of the high molecular weight protein, the ribosomes synthesize coat protein either without leaving the RNA molecule or because the corresponding gene is now translatable.

Competition experiments are in progress to answer this question.

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